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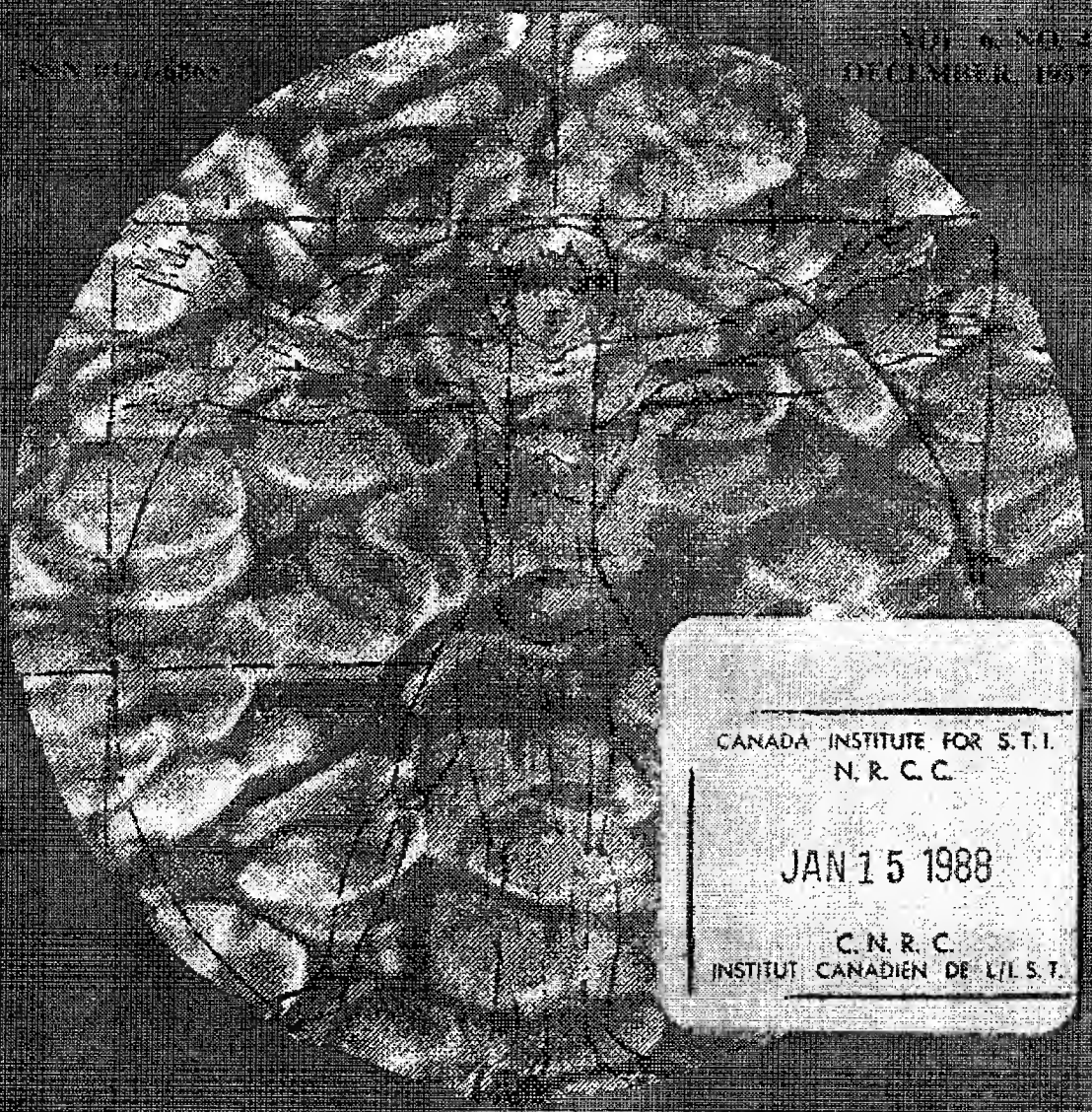

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Quantitation of histamine-induced angiogenesis in the chick chorioallantoic membrane: mode of action of histamine is indirect

W.D. THOMPSON and F.I. BROWN
Department of Pathology, University of Aberdeen, Aberdeen AB9 2ZD, Scotland, UK

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Abstract. Histamine was chosen as a standard chemical suitable for the development of new methods of assessing vascularity in the chick chorioallantoic membrane (CAM). When applied in liquid form to the entire 'dropped' area of CAM at day 10 of growth, a sublethal dose was required before a convincing appearance of increased vascularity was produced by day 15. Computer-assisted morphometry, using osmium-stained CAMs viewed *en face*, demonstrated a progressive increase in both length (21%) and number (59%) of arterial branches over 5 days relative to buffer only controls. A 56% increase in number of mesenchymal vessels seen in cross-section was also detected and this method is the quickest and easiest. No increase in the number of capillaries in the surface plexus of the ectoderm was found but this may be attributable to inadequate technique due to the already high density of capillaries present even in control CAMs using thin resin sections. However a 21% increase in the haemoglobin content of the CAM 4 days after histamine is consistent with an overall increase in the vascular bed. A rapid fall in DNA synthesis by 6 h (−48%) was produced by histamine, followed by a slow rebound to a peak at 36 h (74%). This contrasts with the growth factor type of pattern seen previously with fibrin degradation products giving a progressive rise to a major peak of DNA synthesis at 18 h. These effects indicate two mechanisms of induction of angiogenesis, and that the action of histamine is indirect.

Introduction

Histamine was selected as a commercially available, pure substance, claimed to be angiogenic [26, 8], and therefore suitable for the development of new quantitative methods of assessing vascularity in the chick chorioallantoic membrane (CAM). The lack of truly quantitative methods for such measurement has been commented on by several authors [3, 23, 25]. As an alternative, endothelial cell culture lends itself to quantitation but stimulants of endothelial cell mitosis do not always stimulate angiogenesis in animal models [20]. Chemokinetic and chemotactic substances can be distinguished

by this method [1] but endothelial cell culture has become a complex field in itself and the in vivo relevance of these effects is not yet established. Another difficulty is that the effect of growth factors, such as platelet-derived factor, is dependent on such variables as on the substrate upon which the cells are growing [9, 16]. We have previously shown that the measurement of DNA and protein synthesis in the CAM may elucidate the pathway leading to visible angiogenesis: we have also shown by autoradiography that such assays are not specific for the vascular component [21], hence the need for direct measurement.

Apart from its availability as a standard pure chemical, another reason for the choice of histamine was for comparison with previous work by us showing that fibrin degradation products (FDPs) are angiogenic [21]. Others have shown that certain low molecular weight preparations of FDPs increase vascular permeability, and that this effect can be blocked by the prior application of antihistamine [7]. Therefore it seemed possible that angiogenesis induced by FDPs might also be mediated by histamine, particularly as mast cells are known to be present in the normal CAM [8, 14]. This study shows that the production of angiogenesis by histamine follows an unexpected pathway which does not explain the ability of FDPs to stimulate angiogenesis.

Materials and methods

Dose of histamine

Free base crystalline histamine (Sigma) was dissolved in Dulbecco A buffer and the pH reduced to 7.34 with 1 M HCl and made up to volume. The histamine solution was sterilised by membrane filtration and 0.3 ml aliquots were applied to each chorioallantoic membrane, controls receiving 0.3 ml of Dulbecco A buffer, all being applied at day 10 of chick growth. A range of doses from 0.018 M to 0.27 M were tested and eggs were subsequently examined for changes in the chorioallantoic membrane or used for assay of haemoglobin content or DNA synthesis.

Preparation and use of eggs

Fertile hens' eggs (Ross Brown strain) were prepared, as previously described, by removing 1 ml albumen to allow the formation of artificial air space beneath a window in the side of the shell [21, 22]. This permits the growth of the CAM to form a flat area about 7 cm^2 which could later be

observed through the window. Test or control material is then applied at day 10 of development.

Subsequently, eggs were injected at days 11 to 15 with formalin and, after fixation, the exposed area of CAM was excised and treated in one of two ways. Most of the CAM was stained with 2% osmium tetroxide in phosphate buffer for 3 hours, dehydrated with alcohol, and finally mounted en face on large slides with DPX. These slides were used for computer-assisted morphometry of the arterial vessels. For histological studies the central 3 mm strip of the fixed CAM was excised, washed in phosphate buffer, dehydrated in alcohol, and embedded using a JB-4 embedding system (Polysciences). Thin resin sections ($2\,\mu$) were then cut and stained with Paragon. The number of epidermal capillaries and mesenchymal vessels per mm of CAM cross section was subsequently measured, as was the thickness of the CAM.

Computer assisted morphometry

The slides of CAM mounted en face were projected from a light microscope stage on to a Summagraphics bit-pad linked to a Tectronics 4050 computer. The system was calibrated with a stage micrometer and the magnification used for measurement of arterial branches was × 1440. Each field of view studied corresponded to an area of 1.06 mm^2 of CAM. Osmium staining picks out the arterial branches which retain red cells in the fixed CAM whilst the venous circulation remains unstained. For the determination of length and number of branches of vessels the first order vessel entering the CAM was ignored, as were major second and third order branches. Fourth order branches (less than $10\,\mu$) and further branches (Figs. 1a and 1b) were assessed by tracing with a cursor, allowing subsequent automatic summation of data by the computer.

Assays on the chorioallantoic membrane

The time course of changes induced in DNA synthesis in the CAM by the application of histamine was measured as previously described [21]. Groups of ten control and ten test membranes were assayed at 6 h intervals after the application of control buffer or histamine, respectively, up until 42 hours later. The results of methyl-[^{3}H]-thymidine incorporation were expressed as a percentage of the control mean to exclude the effect of slight diurnal variation in DNA synthesis. Autoradiography with ^{3}H-thymidine was also performed on test and control membranes at 36 h after application. In other experiments the haemoglobin content of the homogenised CAM was meas-

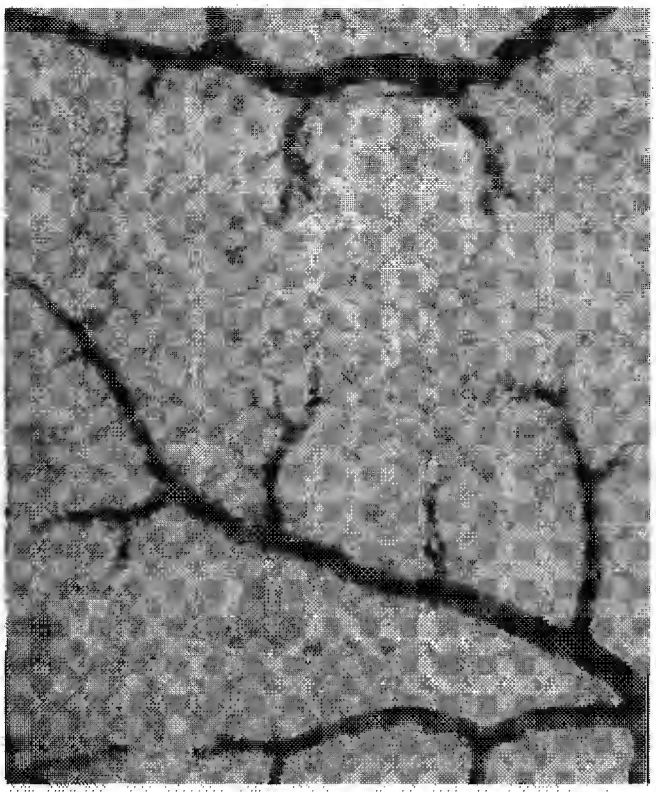

Fig. 1a. The normal straight branching pattern of arterial blood vessels at day 15 in the CAM, stained with osmium and viewed en face. × 60

ured and the result expressed as g haemoglobin per mg Lowry protein. This latter assay was performed as a simple measure of the total vascular bed, accepting the fact that the venous circulation largely empties on excision of the CAM but observing that this occurs in both test and control.

Results

Effective dose of histamine

A range of 7 doses from 0.018 M to 0.27 M were applied to the CAM of groups of 10 eggs. Survival decreased markedly at the dosage of 0.18 M when only 30% of eggs survived from day 10 to day 15. At the dose of 0.09 M, 70% of eggs survived, comparable with lesser dosage and buffer-only controls. On examination of osmium-stained CAMs it was clear that convincing angiogenesis in terms of increased tortuosity and branching was only apparent at the maximum sublethal dose of 0.09 M (10 mg/ml) histamine (Figs. 1a and b). This dose was used for all subsequent work.

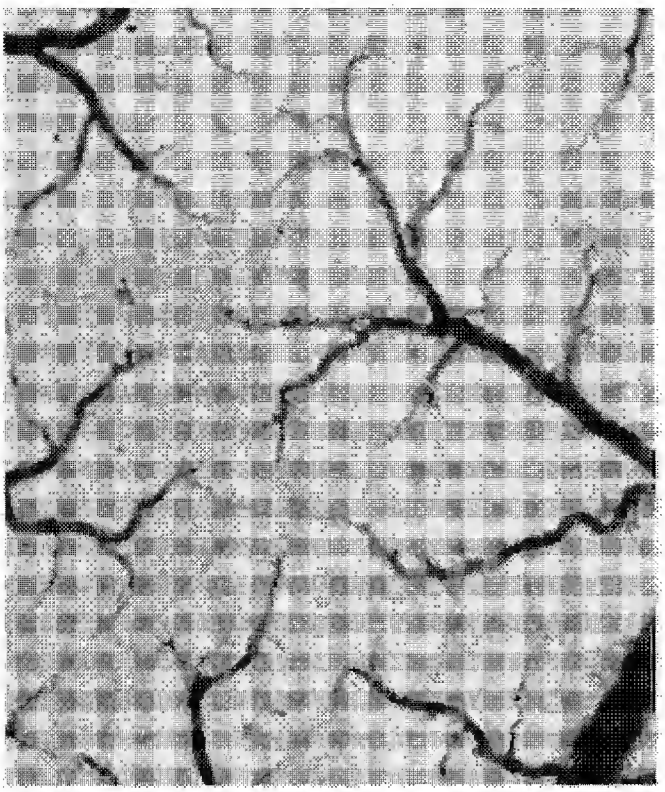

Fig. 1b. In comparison with the previous figure, there is a general increase in vascularity and tortuosity following the application of histamine to flood the surface. ×60

Vascular changes observed by morphometry

By the use of progressive mean estimation [5] it was found that 10 fields of view were sufficient for the purpose of estimating arterial branch length and number and also number of vessel cross-sections in the CAM. Figure 2 shows the progressive increase in total arterial branch length reaching a relative 21% increase by day 15, 5 days after histamine addition. A significant increase in the number of arterial branches also occurred over 5 days amounting to 59% over the control level (Fig. 3). Over the same time a similar increase (56%) was seen in the number of vessels counted in cross-section within the mesenchymal layer (Fig. 4). A dose-response relationship is illustrated for this latter method (Fig. 5).

Despite counting of large numbers of sections and the use of resin processing to obtain thin sections, no convincing increase was seen in the number of capillaries observed in the ectodermal layer. Figure 6a shows the close appostion of capillaries even with normal (control) CAM by day 11 of growth. This dense meshwork of capillaries is viewed en face in Fig. 6b. A transient increase in thickness of 25% due to oedema was apparent on day

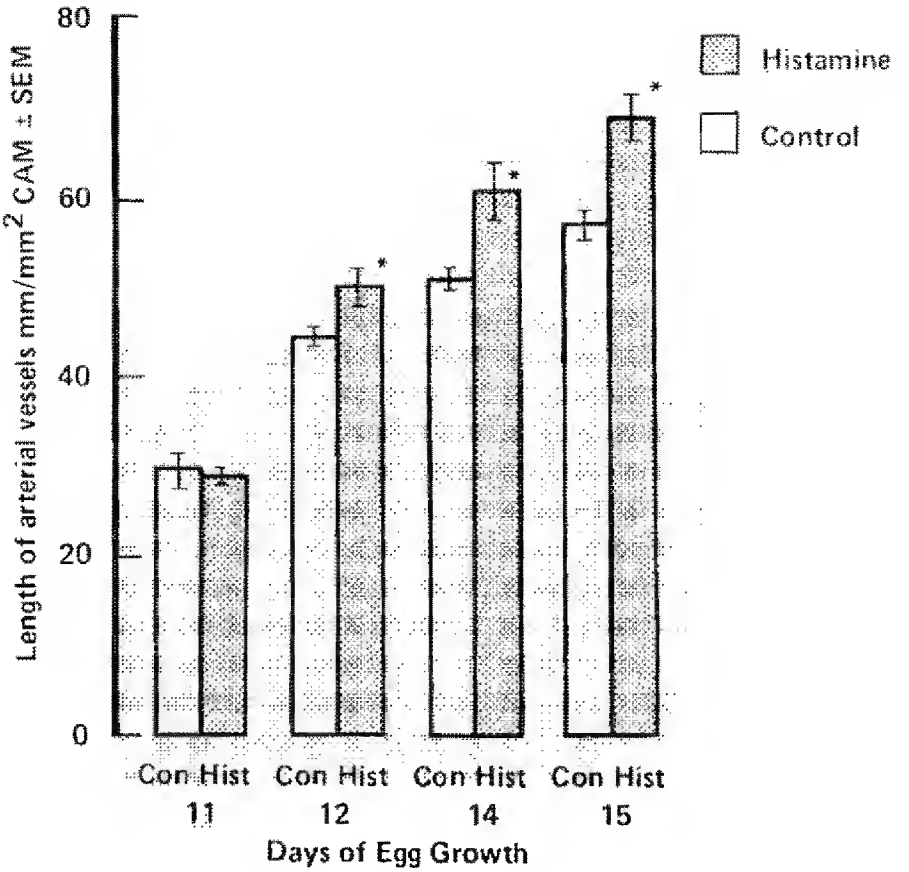

Fig. 2. There is a progressive increase in total arterial branch length reaching a 21% increase over buffer controls by day 15. (* t test, $P < 0.05$)

11, one day after application of histamine, with a return to the control level thereafter.

The haemoglobin content of the CAM (mean ± SEM) after 4 days was 0.095 ± 0.010 g/mg Lowry protein for the control group and 0.114 ± 0.010 g/mg for the histamine group; representing a 20% increase (t test, $p < 0.05$). Following application of histamine, the level of DNA synthesis fell rapidly by 48% at 6 h and then recovered slowly to a peak at 36 h representing a 74% increase (Fig. 7). Autoradiography at 36 h indicates that the increase in [^{3}H]-thymidine labelling is seen in all cell types and layers of the CAM (Fig. 8a and 8b).

Discussion

Focal application has been the usual method of testing potentially angiogenic substances on the chick CAM. However, this method produces the

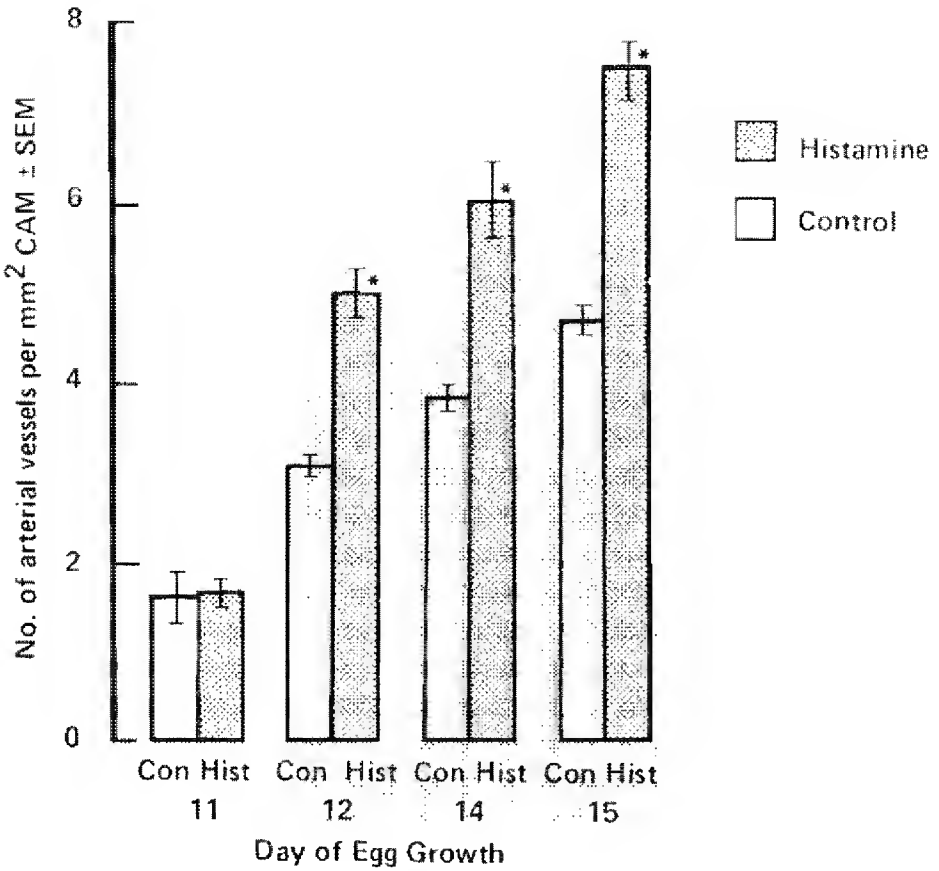

Fig. 3. Over the same time as in Fig. 3, there is a 59% increase in the number of arterial branches.

same spokewheel pattern of radiating vessels whether the substance applied is angiogenic, toxic or physically injurious [13]. Although this pattern of increased vascularity can be quantitated [4, 11, 24], much of this effect could be due to puckering and contraction of the new fibrous tissue component leading to vessel indrawing and not due solely to vascular proliferation [17]. Histological examination will reveal whether the cellular inflammatory response has been evoked, but this is only a prominent feature of certain inflammatory stimuli such as bacterial products, and not necessarily associated with transient increased vascular permeability and oedema [12]. The massive, short-lived increase in permeability induced by histamine has long been known not to be accompanied by a significant leucocyte emigration [10].

By flooding the entire area of CAM that is available we have avoided physical trauma and focal distortion. The surprising result is that a sublethal dose of histamine is required to induce an angiogenic response visible several days after application. Consistent with this, Barnhill and Ryan were not

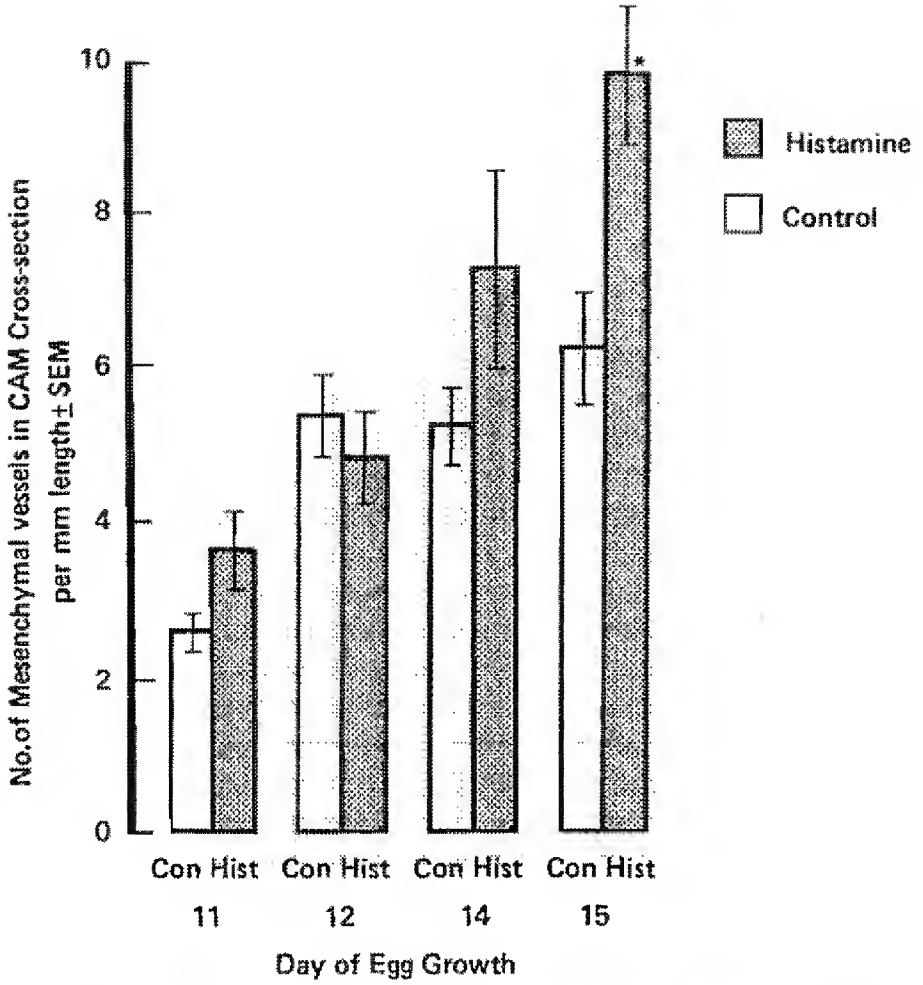

Fig. 4. A similar increase of 56% is apparent in the number of mesenchymal vessels counted in cross-section.

convinced that histamine was angiogenic using focal application at the much lesser dose of 10^{-4} M [4]. It would be wrong to conclude that this effect is irrelevant to in vivo mechanisms. It may be that a similarly high local concentration of histamine is achieved when mast cells release their granules immediately next to small vessels.

The effect of histamine on vascular permeability operates on a time scale of minutes, although the resultant oedema may persist for hours and indeed we have noted persistent oedema histologically in the majority of CAMs after 24 h. In contrast the effect of histamine on vascularity is a progressive one, with a time scale of days. There is an increase after 5 days in length (21%) (Fig. 2) and number (56%) (Fig. 3) of all vessels seen in cross-section in the mesenchymal layer. Estimation of vessel cross-sections has been found to be the most convenient, objective assay and a dose-response relationship for histamine is shown (Figs. 4 and 5). It is apparent that these increases in

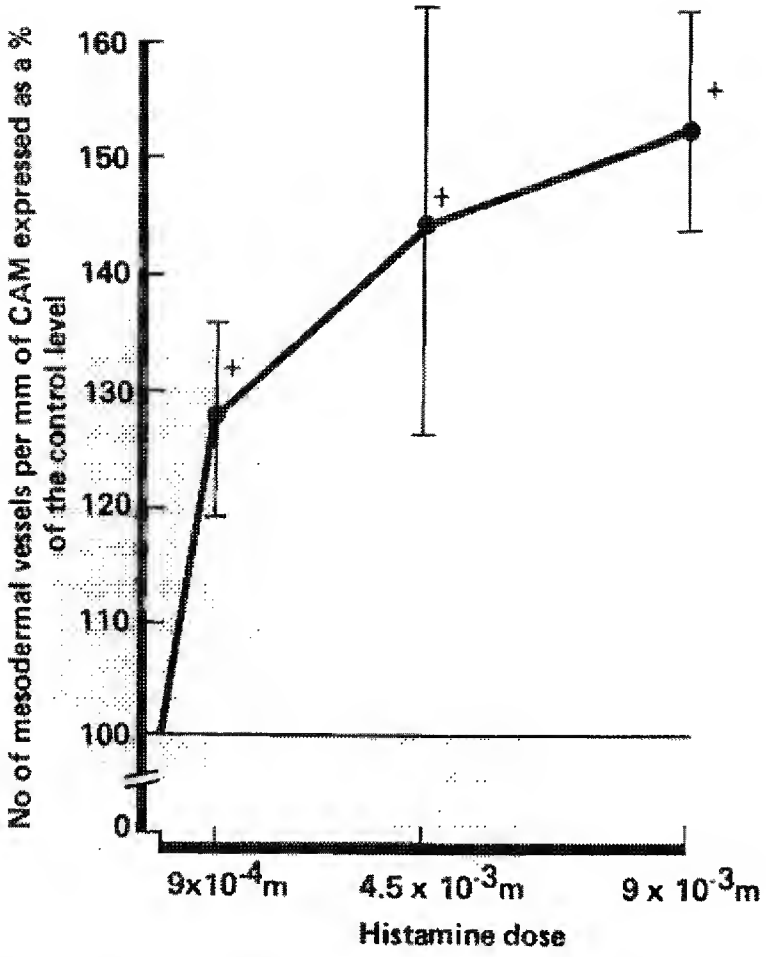

Fig. 5. The effect of increasing concentration of histamine shows that assessment of mesenchymal vessel number can be used to show a dose-response relationship.

vascular structures cannot be accounted for by a transient increase in vascular dilatation. This increase is restricted to the exposed area of CAM and is not present in the adjacent CAM still adherent to the shell, thus excluding any general effect mediated via the entire fetoplacental circulation. The sustained angiogenic effect of histamine applied in liquid form emphasises that focal application of a slow release form of an angiogenic agent is not required to induce new vessels to proliferate over several days.

How does this new vasculature develop? Schoefl (1963) showed that in the healing wound vascularisation occurred both by capillary sprouts and elongation of capillary loops [18]. The intact CAM is an uninjured tissue, however, and recently Schoefl (1984) showed that arterial and venous branches evolve from preferred channels of flow in the dense capillary plexus already present by day 6 in the normal CAM [19]. Considering that the chick embryo and its entire vasculature has just 20 days to mature before hatching,

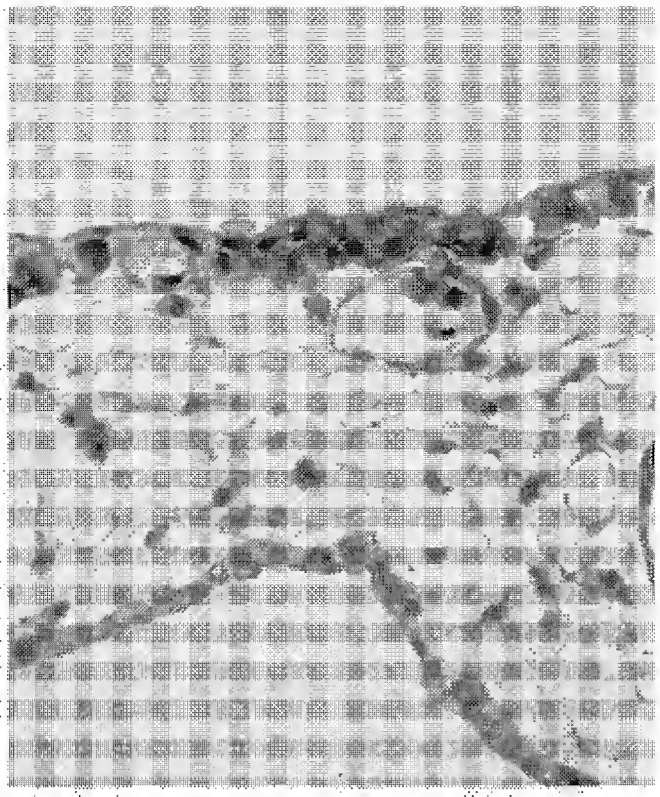

Fig. 6a. Capillaries containing nucleated red cells are closely apposed within the surface ectodermal layer in a normal CAM at day 11 of growth. Paragon ×248

it is not surprising that stimulation of the CAM produces new arterial and venous branches over a five-day period. Histamine has been claimed to be mitogenic to cultured capillary endothelial cells [15]. Our inability to demonstrate an increase in capillaries is presumed to be due to technical inadequacy (Figs. 6a and b) since the increase in haemoglobin content observed indicates an overall expansion of the vascular bed.

The rapid, profound fall in DNA synthesis in the CAM after histamine application may be considered just a 'toxic' effect but it is presumably this effect which initiates angiogenesis. It may be that the increase in vascular permeability and throughput of tissue fluid removes ^{3}H-thymidine from the site of application even more quickly than normal, thus obscuring the true rate of incorporation at cellular level. However, it may equally be argued that this would reflect the reality of what is happening to endogenous nucleotides and amino acids. In any case, we have observed that there is an apparent rise in DNA synthesis at 36 h (Fig. 7) associated with [^{3}H]-thymidine labelling of all major cell types (Figs. 8a and b) and this contrasts with the decline of DNA synthesis after the peak at 18 h induced by FDPs [21]. This delay suggests that FDPs do not act via the stimulation of histamine release by mast cells in the CAM. The effect of histamine on

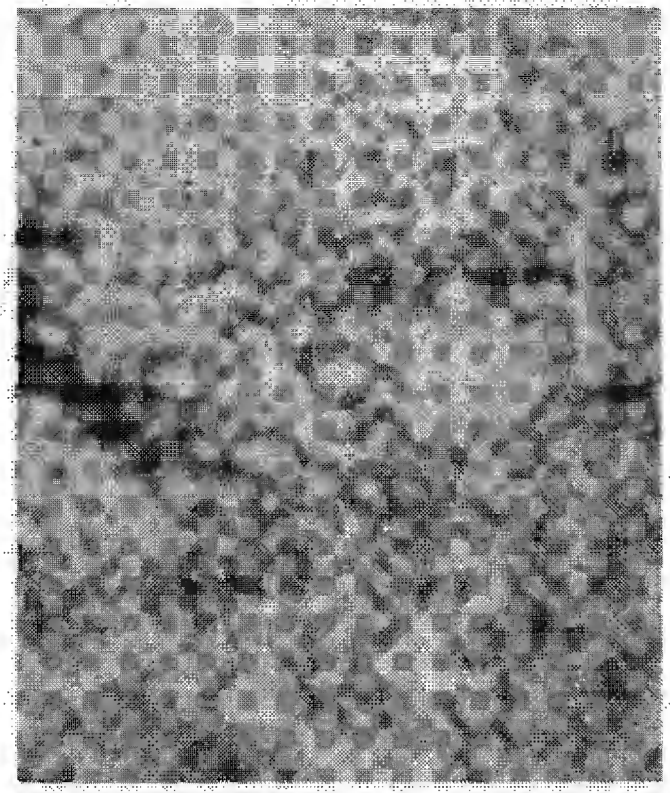

Fig. 6b. This dense meshwork of capillaries is viewed en face in an osmicated CAM. A terminal arterial branch (out of focus) in the underlying mesenchyme is seen to divide to supply the capillary plexus. × 297

vascular permeability is mediated by prostaglandin synthesis [2] but this is a rapid phenomenon which does not account for the delay before onset of increased DNA synthesis.

This delay is interesting even if it is regarded as a 'toxic' effect. It may be that many substances claimed to be angiogenic actually act in this way, and it would now be of interest to examine other vasoactive substances such as serotonin and 5-hydroxytryptamine, also held to be angiogenic [8]. In addition to FDPs, we have found that tumour angiogenic factor (crude, native material from Landschütz ascites tumour) stimulates DNA synthesis by over two-fold at 18 h after application (data not shown). The major growth factors stimulate serum-arrested cultured cells to reach a peak of DNA synthesis at around 18 h. This suggests that FDPs may also have a direct effect on cells in G_o phase of the cell cycle in the CAM. Histamine appears to act at an earlier point in the in vivo mitogenic/angiogenic pathway. Increased vascular permeability will result in increased leakage of plasma proteins including fibrinogen. There is recent evidence that such extravascular fibrinogen (elicited with histamine in the rat) is entirely converted to fibrin [6]. We would speculate that it is the gradual degradation of

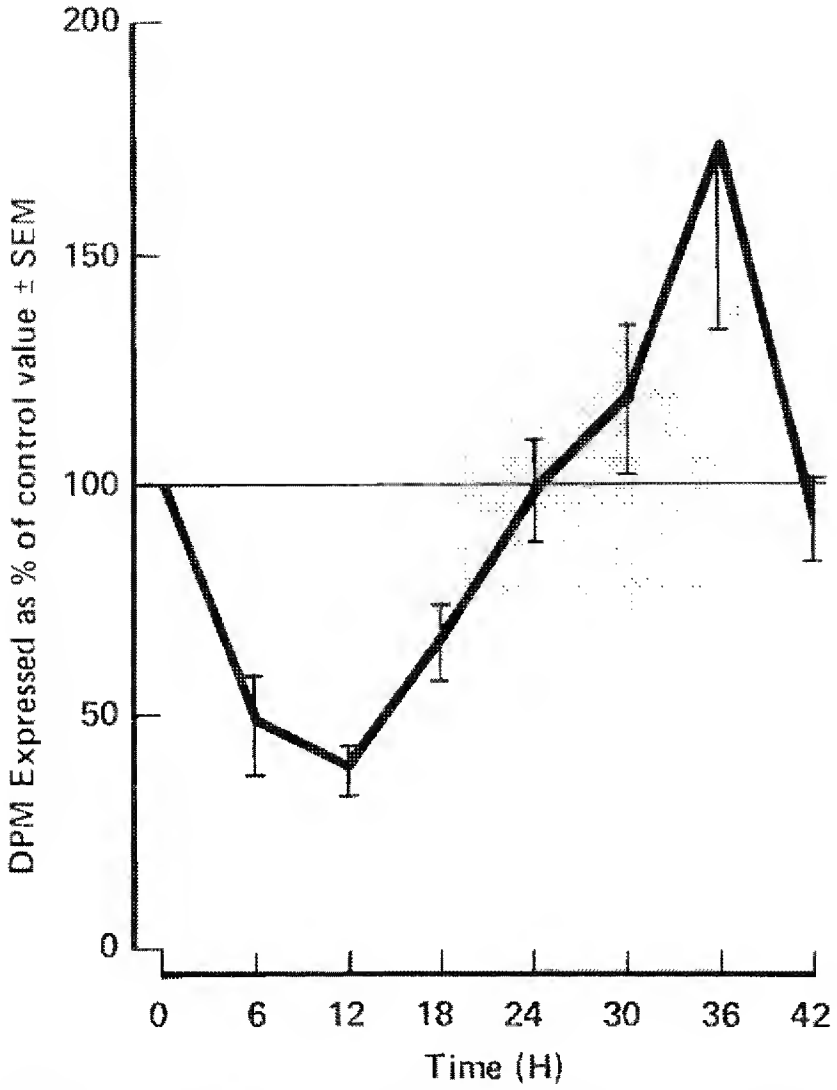

Fig. 7. The effect of histamine on DNA synthesis in the CAM. There is a rapid drop in level of synthesis with a slow recovery to a peak at 36 hr which is 74% above the control level.

endogenous extravascular fibrin which stimulates the next step in histamine-induced angiogenesis.

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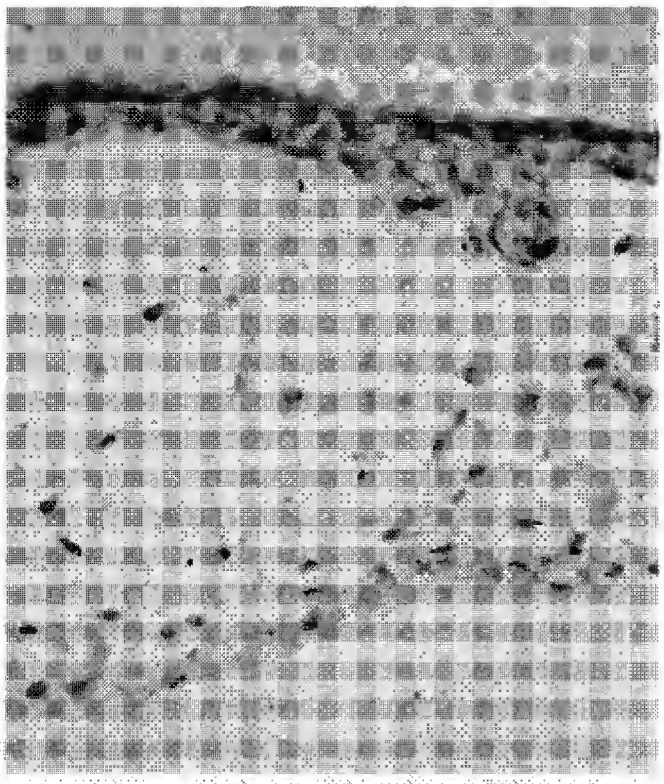

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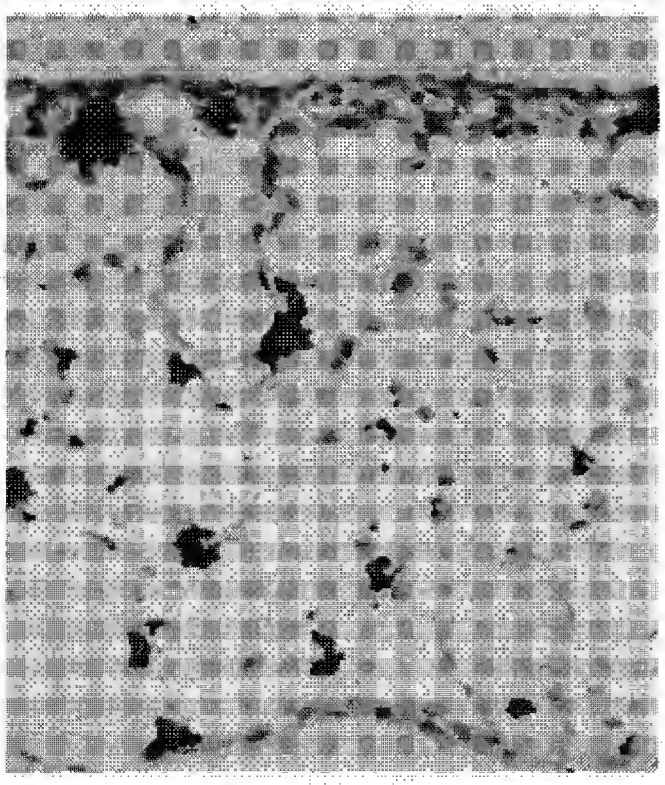

b.

Fig. 8a. Autoradiography with [^{3}H]-thymidine at 36 h after application of control buffer only. × 248

Fig. 8b. Autoradiography of the CAM at 36 h after the application of histamine. Increased labelling of cell types in all layers of the CAM is seen. × 248

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Address for offprints: Dr W.D. Thompson, Department of Pathology, University Medical Buildings, Aberdeen AB9 2ZD, United Kingdom